MECHANISMS OF DEVELOPMENT XXX (2008) XXX–XXX

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Specific and effective gene knock-down in early chick embryos using morpholinos but not pRFPRNAi vectors

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ARTICLE INFO

Article history:
Received 30 May 2008
Received in revised form
21 August 2008
Accepted 25 August 2008

Keywords:
Apoptosis
Dicer
Drosha
Electroporation
Gene silencing
Ear
Otic
p53
Placodes
Preplacodal region
shRNA

ABSTRACT

In the chick embryo, two methods are now used for studying the developmental role of genes by loss-of-function approaches: vector-based shRNA and morpholino oligonucleotides. Both have the advantage that loss-of-function can be conducted in a spatially and temporally controlled way by focal electroporation. Here, we compare these two methods. We find that the shRNA expressing vectors pRFPRNAi, even when targeting a non-expressed protein like GFP, cause morphological phenotypes, mis-regulation of non-targeted genes and activation of the p53 pathway. These effects are highly reproducible, appear to be independent of the targeting sequence and are particularly severe at primitive streak and early somite stages. By contrast, morpholinos do not cause these effects. We propose that pRFPRNAi should only be used with considerable caution and that morpholinos are a preferable approach for gene knock-down during early chick development.

1. Introduction

Sequence specific knock-down strategies using morpholino oligonucleotides and short-interfering RNAs (siRNA) have proven to be powerful tools to study gene function in different organisms. However, sequence-independent off-target effects have been reported in mammals and fish to cause developmental defects including wide spread cell death (Ekker and Larson, 2001; Robu et al., 2007; Scacheri et al., 2004). The chick embryo is a well established developmental model system, which lends itself particularly well to the introduction of DNA constructs by electroporation into specific tissues to perform temporally and spatially controlled gain- and loss-of-function studies (Funahashi et al., 1999; Katahira and Nakamura, 2003; Muramatsu et al., 1997; Nakamura et al., 2004, 2000).

For loss-of-function, electroporation of morpholinos (Kos et al., 2003; Papanayotou et al., 2008; Sheng et al., 2003; Tucker, 2001; Voiculescu et al., 2007) or expression of dominant negative DNA constructs (Becker et al., 2001; Bel-Vialar et al., 2002; Chen and Cepko, 2002) have both been successful. Recently, methods for vector-based RNA interference have been described, requiring small amounts of DNA to be introduced into tissues, while allowing long lasting and stable expression

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0925-4773/$ - see front matter
doi:10.1016/j.mod.2008.08.005

of the short-interfering RNA (Bron et al., 2004; Das et al., 2006; Katahira and Nakamura, 2003). One approach in particular used a system to express short-hairpin RNA molecules, which resemble naturally occurring microRNA (miRNA) and appear to be processed efficiently by endogenous enzymes to yield siRNA and gene silencing (Das et al., 2006). Most studies using siRNA approaches generally investigate processes that occur after the 10-somite stage, while morpholino knock-down has most successfully been used in young embryos before or around primitive streak stages.

To establish a reliable loss-of-function strategy in young chick embryos we compared the vector-based approach using short-hairpin RNAs (shRNA; in particular pRFPRNAi; Das et al., 2006) with morpholinos. We were surprised to find that vectors targeting Eya2, Pax2 and Notch1 as well as control vectors targeting GFP, cause identical morphological phenotypes including the absence of cranial placodes and neural tube closure defects, and mis-regulation of neural, preplacodal, otic and olfactory placode markers. In addition, elevated levels of apoptosis are observed. These non-specific effects appear to be independent of the miRNA pathway member Dicer or of activation of the interferon pathway, but seem to be due to activation of p53 target genes. To determine whether all antisense methods suffer from the same shortcomings, we carried out similar experiments using morpholinos and find that they cause efficient and specific knock-down, but do not show non-specific side-effects. Our data suggest that using morpholinos is the preferential approach for gene knock-down in early (HH4–10) chick embryos.

2. Results

2.1. pRFPRNAi gene silencing vectors affect otic morphology and gene expression

Because of our interest in ectodermal patterning and sensory placode formation, we focussed on designing knock-down strategies for genes that are expressed in the placode territory (*Eya2*) and the otic placode (*Pax2*). We started by using shRNA expression vectors pRFPRNAi (Das et al., 2006) and used the otic placode, which becomes morphologically discernible at stage HH10, as a read out.

The *Eya2*-targeting vector was introduced into stage HH5-6 embryos. Embryos were incubated overnight, harvested and tested for *Pax2* expression as the earliest otic marker (Groves and Bronner-Fraser, 2000). RFP^+ cells expressing the *Eya2* silencing vector have reduced *Pax2* expression and otic placode morphology is severely disrupted (6/6; Fig. 1A, A′ and a). To test the efficiency of the silencing vector, we performed *in situ* hybridisation using a 3′UTR probe for *Eya2*. Surprisingly, expression of the *Eya2* silencing vector causes up- rather than down-regulation of the transcript (6/7; Fig. 1B, B′ and b). This unexpected result could be caused by off-target effects of the *Eya2*-target sequence in the silencing vector. To test this, we introduced silencing vectors targeting the otic specific genes *Pax2* and *cNotch1* (Adam et al., 1998; Daudet and Lewis, 2005; Groves and Bronner-Fraser, 2000). The *Pax2* silencing vector causes disruption of otic cup morphology and only few cells retain expression of *Pax2* ($n = 19/19$; Fig. 1C and C′; Table 1) or the otic markers *BMP7* and *GATA3* (13/13 and 10/10, respectively; not shown). A similar effect is observed when *cNotch1* is targeted: *cNotch1* expression is reduced (6/6; Fig. 1D and D′), *Pax2* expression (6/8; Fig. 1E and E′) and otic morphology is lost (Fig. 1e). Although these effects may suggest an early role for both genes in otic development, the loss of *Pax2* transcripts and the otic placode after pRFPRNAi *cNotch1* expression is unexpected, because previous studies showed that Notch inhibition by the γ-secretase inhibitor DAPT leaves otic structures largely unaffected (Abello et al., 2007). Furthermore, the consequences of targeting *Pax2* and *cNotch1* strongly resemble the effects of the *Eya2* silencing vector. Consistent with this, we also observe an up-regulation of *Eya2* after pRFPRNAi *Pax2* expression (14/14; not shown).

The above results suggest that the effects of pRFPRNAi vectors are non-specific. To test this, the presumptive otic ectoderm on one side of the embryo was electroporated with a vector targeting GFP (Das et al., 2006) together with pCAβ-IRES-GFP (driving GFP ubiquitously). The contralateral side of the same embryo received pCAβ-IRES-GFP only (Fig. 1F). The side expressing GFP alone contains fluorescent cells (Fig. 1F′) and the otic cup is intact expressing *Pax2* (bent arrow in Fig. 1F″), while the side transfected with both GFP and the silencing vector shows greatly reduced fluorescence. However, no *Pax2* (0/20; Fig. 1F″ and 1g; Table 1) or *cNotch1* (0/5; not shown) expression is detected and otic morphology is lost completely (Fig. 1g). In contrast, neither misexpression of vectors encoding GFP or RFP ($n \geqslant 40$ each) nor coexpression of both fluorescent proteins impaired otic development ($n = 5$ using pCAGGS vectors; $n \geqslant 60$ using pCAβ-IRES vectors).

To rule out the possibility that the phenotypes observed are specific to otic markers and morphology, we investigated whether the silencing control vector pRFPRNAi *GFP* also alters the expression of preplacodal, neural plate and placode markers (Table 1). We observe ectopic expression of *Sox3* (14/19; Fig. 2E and E′), *Six1* (7/11; Fig. 2D and D′), *Six4* (8/14; Fig. 2F, F′ and f), and *RALDH3* (3/7; not shown) and occasionally, with the exception of *Sox3*, loss of endogenous gene expression. Reduced *Sox2* expression in the neural plate appears to be a consequence of changes in neural tube morphology rather than loss of gene expression (Fig. 2C, C′ and c). Finally, we see down-regulation of *Eya2* expression (Fig. 2B, B′ and b) when using a full length – rather than a 3′UTR-antisense probe (Fig. 2A, A′ and a). We cannot fully explain the discrepancy between the results when using two different *Eya2* probes. We can rule out hybridisation of the 3′UTR probe to sequences in the electroporation vector: probes for *Dlx5* 3′UTR, *Eya2* FL and 3′UTR, *GnRH1*, *cNotch1*, *Pax2* 3′UTR and FL, *Sox2* and *Sox3* were synthesised using the same plasmid template and include the same or similar sequences between the RNA polymerase promoter and the multiple cloning site of the template vector. Following pRFPRNAi electroporation, only some of these genes are up-regulated, while others are not. This excludes the possibility that the results are due to binding of the probes to the vector sequences. One possible explanation is that by introducing the silencing vectors short transcripts are created to which the shorter *Eya2* 3′UTR, but not the longer *Eya2* FL probe can bind. All phenotypes described are independent of the silencing vector concentration being still observed with as little as 300 ng/μl vector. At this

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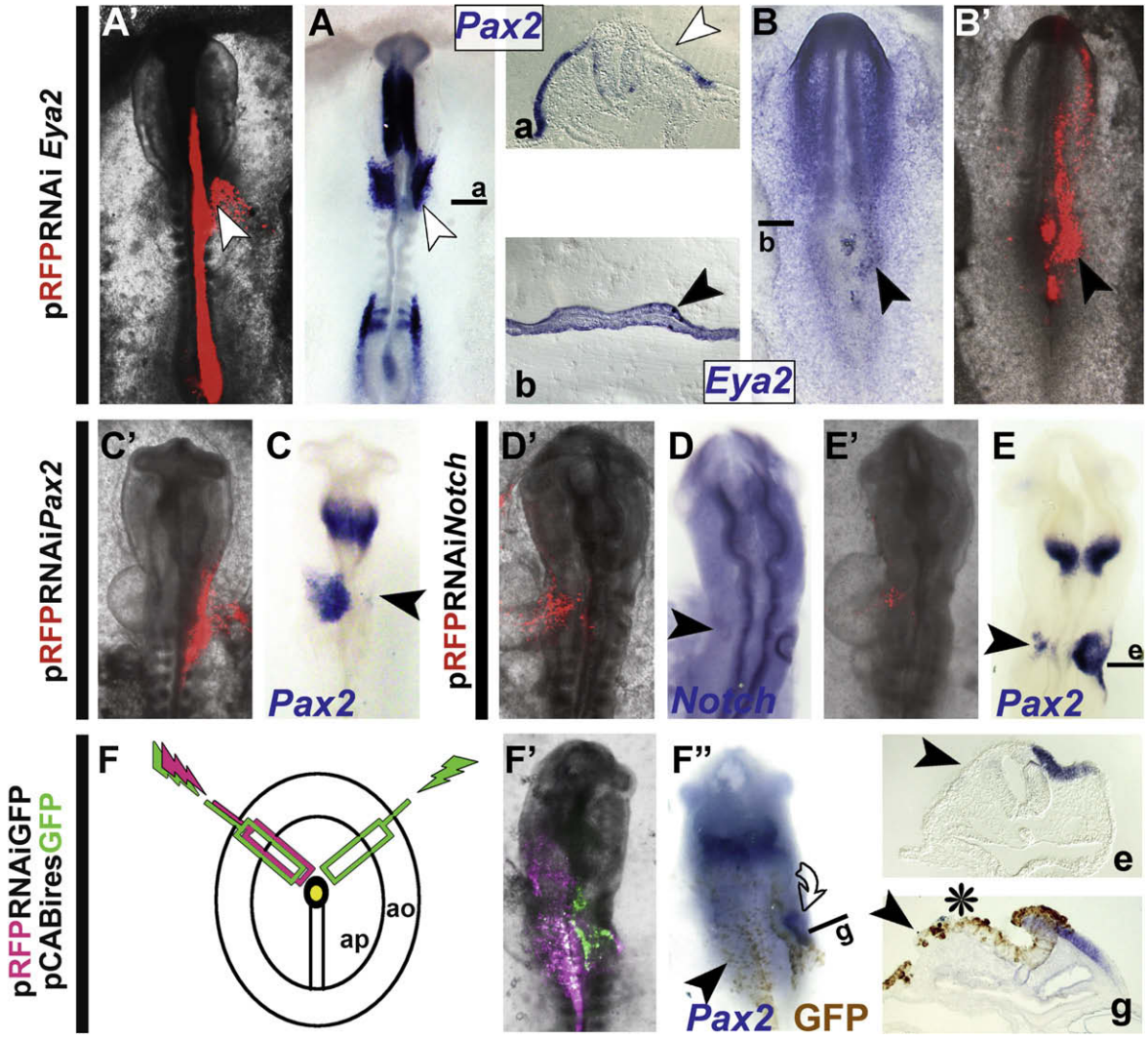

Fig. 1 – Expression of gene silencing vectors causes loss of otic markers and otic cup morphology, independent of the target sequence. (A, A′) Expression of Eya2 pRFPRNAi as revealed by the presence of RFP (A′) causes down-regulation of otic *Pax2* expression (arrowhead, A and transverse section a). (B, B′). Up-regulation of *Eya2* rather than loss of expression is observed after misexpression of the Eya2 pRFPRNAi vector (arrowhead in B, B′; transverse section b). In A and B black bars indicate level of sections shown in a and b. (C–E′) Expression of silencing vectors targeting *Pax2* (C′, red) and *cNotch1* (D′, E′, red) results in loss of *Pax2* and *cNotch1* expression (arrowheads in C–E). Down-regulation of otic markers is accompanied by loss of otic cup morphology (arrowhead in E and e). (F–g) GFP expressing vector was electroporated together with a GFP silencing pRFPRNAi into the presumptive otic territory on one side of the embryo (F, left; F′, magenta and white), while the contralateral side was electroporated with GFP expressing vector only (F, right; F′, green). Otic tissue expressing GFP alone shows normal placode morphology and *Pax2* expression (bent arrow in F″). GFP expression is reduced where GFP expressing and GFP silencing vectors were coelectroporated (F′, F″). Additionally, *Pax2* expression is lost, no otic placode is visible (arrowhead in F″ and g) and neural tube closure is impaired (* in g). Side-effects can be observed irrespective of concentrations used for electroporations (A′, B′ and F′, 1 μg/μl; C′ and D′, 0.5 μg/μl; E′, 0.3 μg/μl). ap, area pellucida; ao, area opaca. The section planes in e and g are indicated by small bars in E and F″, respectively.

concentration vector-based RFP expression can barely be detected (Fig. 2E).

2.2. *Morpholino oligonucleotides do not cause non-specific effects*

The above results suggest that pRFPRNAi vectors cause non-specific effects. To test whether this is the case for other antisense methods for knocking-down gene expression, we designed two morpholinos targeting intron–exon boundaries of *Eya2* and two morpholinos targeting *Pax2*. Both *Eya2* morpholinos were electroporated simultaneously into stage HH4–5 embryos, targeting the presumptive otic epithelium (Garcia-Martinez et al., 1993; Streit, 2002). Their efficiency and specificity was confirmed by RT-PCR of electroporated tissues (Fig. 3A). Neither morpholinos targeting *Eya2* ($n = 18$) nor control ($n = 14$) morpholinos affect the expression of *Pax2* or otic morphology (Fig. 3B). Electroporation of the Pax2

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Table 1 – Effects after electroporation of gene silencing vectors or morpholinos into early chick embryos

	Ectopic expression	No effect	Loss of expression	*n*
ISH probe				
pRFPRNAi Eya2				
Eya2 3′UTR	6	1	0	7
Pax2	0	0	6	6
pRFPRNAi *Pax2*				
Pax2	0	0	19	19
BMP7	0	0	13	13
GATA3	0	0	10	10
Eya2 3′UTR	14	0	0	14
Eya2 FL	0	2	5	7
pRFPRNAi *Notch1*				
Notch1	0	0	6	6
Pax2	0	2	6	8
pRFPRNAi *GFP*				
Eya2 3′UTR	22	0	0	22
Eya2 FL	0	7	7	14
Sox2	0	5	16*	21
Sox3	14	5	0	19
Six1	7	4	2	11
Six4	8	6	2	14
Pax2	0	0	20	20
Pax2 3′UTR	0	0	7	7
Notch1	0	0	5	5
RALDH3	3	2	2	7
GnRH	0	27	1	28
Dlx5 3′UTR	0	5	0	5
TUNEL staining	Cell death	No cell death	Unrelated cell death	
	12	7	7	26
ISH/Ab probe				
Eya2 morpholino				
Eya2 FL	0	9	0	9
Six1	0	15	0	15
Six4	0	8	0	8
Pax2	0	18	0	18
Pax6	0	10	0	10
Pax2 morpholino				
Eya2 3′UTR	0	8	0	8
Eya2 FL	0	6	0	6
Six1	0	7	0	7
Sox3	0	6	0	6
Pax2	0	0	12	12
Pax6	0	4	0	4
Dicer morpholino				
Eya2 3′UTR	0	13	0	13
Eya2 FL	0	17	0	17
Pax2	0	6	0	6
pCAβ-IRES-GFP/RFP				
Pax2	0	⩾60	0	⩾60
TUNEL staining	Cell death	No cell death	Unrelated cell death	
	0	8	3	11

Electroporation of different gene silencing vectors causes ectopic expression and/or loss of preplacodal, placodal or neural markers. These effects are not observed when morpholinos or vectors ubiquitously expressing GFP and/or RFP are electroporated. Ab, antibody; * the apparent loss of *Sox2* expression is due to impaired neural tube closure. Unrelated cell death refers to embryos, where elevated apoptosis was observed outside the electroporated area.

morpholinos individually or combined efficiently reduces Pax2 protein as determined by antibody staining (Fig. 3D–D″, F and G″), while cells carrying sense or standard control morpholinos express Pax2 normally (Fig. 3E–E″ and H–H″). In addition, loss of Pax2 leads to thinning of the otic placode suggesting that Pax2 may be required for its normal morphology. We expanded this analysis to other molecular markers and find that, unlike misexpression of pRFPRNAi vectors, electroporation of Eya2 or Pax2 morpholinos does not lead to ectopic up-regulation of *Eya2* (Eya2-MO: $n = 9$; Pax2-MO: $n = 8$), *Six1* (Eya2-MO: $n = 15$; Pax2-MO: $n = 7$), Pax6 (Eya2-MO: $n = 10$; Pax2-MO: $n = 4$), *Six4* (Eya2-MO: $n = 8$) or *Sox3* (Pax2-MO: $n = 6$; Fig. 3C and C′).

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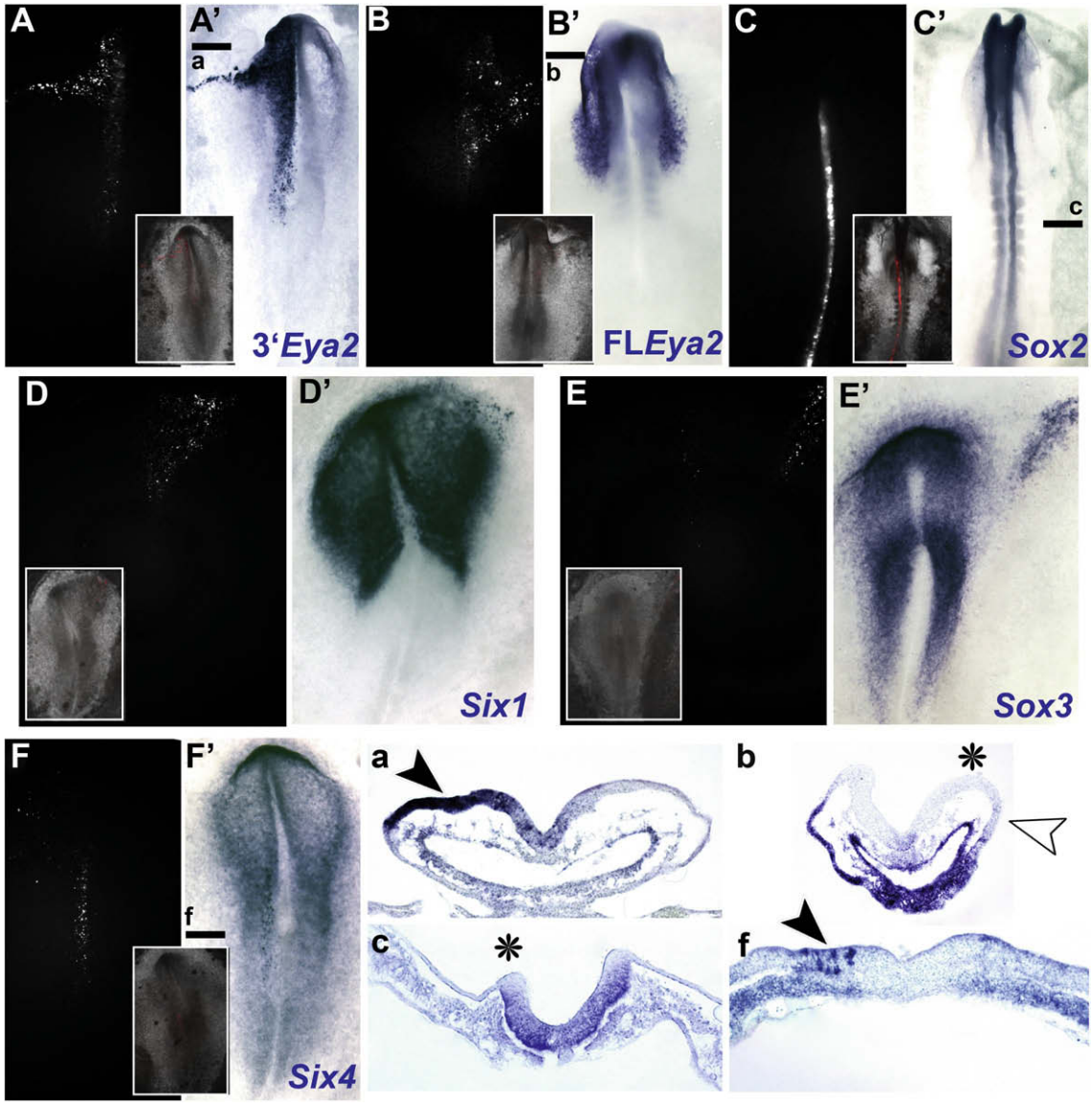

Fig. 2 – Side-effects caused by the expression of gene silencing vectors. The expression of silencing vectors targeting GFP causes up-regulation (black arrowheads in a and f) or down-regulation (white arrowhead in b) of the PPR markers *Eya2* (A, A′, a, B, B′, b), *Six4* (F, F′, f) and *Six1* (D, D′) and the neural marker *Sox3* (E, E′). The apparent loss of the neural marker *Sox2* (C, C′, c) is due to malformation of the neural tube (*, see also b and Fig. 1g). Note that expression of the GFP silencing vectors causes up-regulation of *Eya2* if tested with an *in situ* hybridisation probe against the 3′UTR (3′*Eya2* in A′), but a loss of *Eya2* when using a full length probe (FL*Eya2* in B′). All embryos were incubated for 6 h after electroporation and therefore show only weak reporter RFP expression (A–F; insets show RFP fluorescence and bright field image for orientation). Bars indicate section planes.

Taken together, the above results indicate that morpholinos can be used for specific knock-down of gene function in early chick embryos, whereas pRFPRNAi vectors cause non-specific effects including up- and down-regulation of head ectoderm markers and defects in otic placode formation.

2.3. *Expression of Dicer, Drosha and Argonaute2*

The RNA interference system used allows optimal processing of the expressed transcripts by the endoribonucleases Drosha and Dicer (Das et al., 2006), which should yield better silencing efficiency than conventional RNAi approaches (Chang et al., 2006). If these enzymes are not expressed in relevant tissues, the overexpressed transcripts may be processed inappropriately or not at all, which in turn can result in unspecific cellular responses (Sledz et al., 2003). To test whether this might be the cause of the non-specific effects observed, we analysed the expression patterns of both endoribonucleases and a component of the RNA-induced silencing complex (RISC) *Argonaute2* (Hammond et al., 2001) between stages HH5 and HH12.

At neural plate stages (HH5–HH7, Fig. 4), *Dicer* and *Drosha* are strongly expressed throughout the ectoderm and weakly in the mesoderm, but appear to be absent from the endoderm

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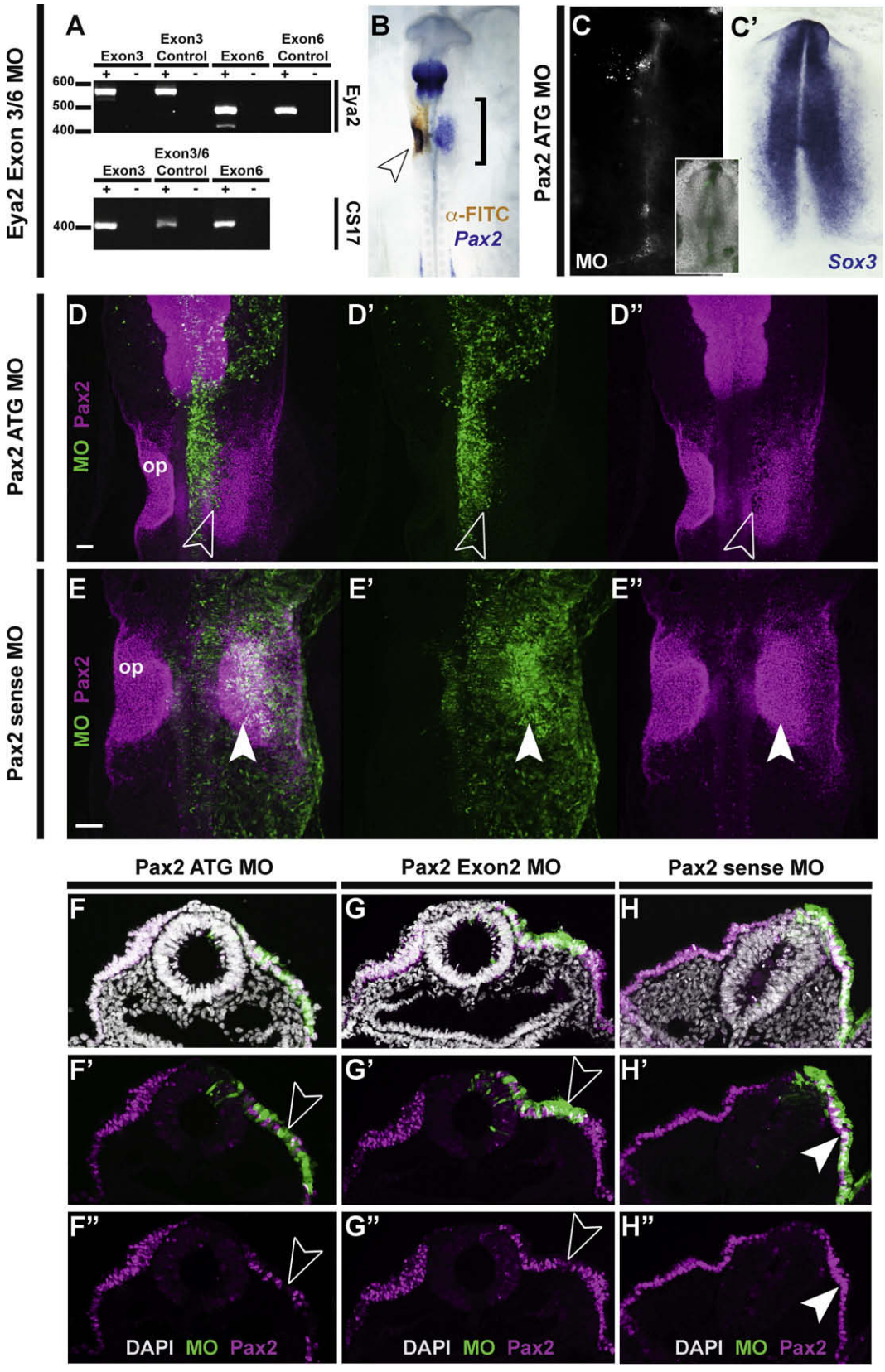
(Fig. 4A–b‴ and D–e‴). Likewise, *Argonaute2* is detected throughout the ectoderm in stage HH5–7 embryos (Fig. 4C–c‴ and F–f‴) but is absent from both mesoderm and endoderm. At placode stages (HH9–10) *Dicer* is expressed in the neural tube, neural crest cells and weakly in the head ectoderm (Fig. 4G, g′–g^{v}) as well as in the prechordal (Fig. 4g′ and g″), somitic (Fig. 4g^{v}) and lateral plate mesoderm (Fig. 4g″^{v}) and the developing heart (Fig. 4g‴). *Dicer* is not present in the notochord or paraxial head mesoderm (Fig. 4g′–g‴). The expression of *Drosha* and *Argonaute2* differs substantially



from *Dicer* (Fig. 4G–i^{v}). Both are detected exclusively in the ventral and lateral aspects of the neural tube and faintly in the open neural plate. No expression is observed in the mesoderm or endoderm.

Differential expression of *Dicer*, *Drosha* and *Argonaute2* is also observed at stage HH12 (Fig. 4J–k$^{v\prime}$). *Dicer* transcripts are found in the otic cup and the ectoderm ventral to it (Fig. 4j$^{\prime v}$), in the neural tube with strongest expression dorsally (Fig. 4j′–j$^{v\prime}$), in the optic vesicle (Fig. 4j′) and in migrating neural crest cells (Fig. 4j″, j‴ and 4j^{v}). Furthermore, *Dicer* expression can also be seen in the ventral wall of the foregut (Fig. 4j‴–j^{v}), the heart (Fig. 4j^{v}) and weakly in the dermomyotome (Fig. 4j$^{v\prime}$), while it remains absent from the head mesoderm (Fig. 4j′–j‴). In contrast, expression of both, *Drosha* (Fig. 4K–k$^{v\prime}$) and *Argonaute2* (not shown) can only be detected in the ventral and lateral neural tube, the optic stalks and weakly in the developing heart.

Thus, while *Dicer* is expressed in all tissues where gene silencing vectors were tested, *Drosha* and *Argonaute2* are only present in preplacodal ectoderm and the neural tube. It has been proposed that Dicer is the limiting factor in the RNAi pathway (Duchaine et al., 2006; Mikuma et al., 2004) and that it can associate with distinct classes of RISC to repress mRNA expression (Forstemann et al., 2007; Okamura et al., 2004; Tomari et al., 2007). Hence, the finding that *Dicer* is expressed in the relevant tissues suggests that absence of this enzyme is not the reason for the non-specific effects of the gene silencing vectors.

2.4. *Side-effects caused by pRFPRNAi expression are independent of Dicer activity*

It is possible that transcripts from the ectopically expressed gene silencing vectors compete with endogenous small RNAs for processing by Dicer. If Dicer is the limiting factor in the RNAi pathway (Duchaine et al., 2006; Mikuma et al., 2004), expression of the gene silencing vectors may lead to insufficient processing of endogenous substrates and the absence of miRNAs that normally keep specific genes silent. As a consequence, changes in unrelated transcripts may be observed. To test this hypothesis we investigated whether morpholino-mediated knock-down of Dicer leads to mis-regulation of gene expression. Embryos electroporated with morpholinos that interfere with *Dicer* splicing events (Fig. 5C) were examined for *Eya2* or *Pax2* expression. Unlike the striking effects observed after electroporation of silencing vectors, reduction of *Dicer* did not affect *Eya2* (Fig. 5A, A′ and a for *Eya2* 3′UTR, 13/13; Fig. 5B, B′ and b for *Eya2* full length, 17/17) or *Pax2* expression or otic cup morphology (6/6; not shown). It is therefore unlikely that the expression of the gene silencing vectors results in up- or down-regulation of gene expression by sequestering endogenous Dicer. Moreover, these results support the previous findings that electroporation of morpholinos in general does not cause similar side-effects.

We further tested whether the expression of the gene silencing vectors affects Dicer expression levels: no changes were observed by RT-PCR (Fig. 5D, control primers) or by *in situ* hybridisation (not shown). To assess whether the side-effects observed are dependent on Dicer activity, we electroporated GFP silencing vectors together with Dicer morpholinos. However, all embryos transfected with both silencing tools showed the characteristic unspecific phenotypes described above (10/10; not shown). These results suggest that the side-effects caused by the pRFPRNAi expression are not due to competition for Dicer.

2.5. *Interferon responsive genes are unaffected by pRFPRNAi GFP expression*

The interferon pathway functions as an initial defence mechanism against viral infection. Activation of this innate immune response is triggered partly by dsRNA, a common viral replicative intermediate (Sledz et al., 2003). The resulting signalling cascade is mediated by a variety of proteins culminating in the induction of interferon-stimulated genes (ISGs; Pebernard and Iggo, 2004; Sledz et al., 2003; Witting et al., 2008). Although unspecific activation of the interferon system through expression of silencing RNAs has so far not been reported in chick, in mammalian cells ISGs can be activated independently of interferon ligand (Sledz et al., 2003; Sledz and Williams, 2004). *p56* and *FGF2* are two classic ISGs that

Fig. 3 – Morpholino mediated knock-down in early chick embryos. (A) Two different morpholinos were used to reduce Eya2 expression by targeting exon–intron boundaries: the first morpholino results in the deletion of exon 3, revealed by an amplicon 49 bp smaller than wild type (top row, lane 1). The second morpholino causes excision of exon 6 resulting in a band 68 bp smaller than wild type (top row, lane 5). The wild type transcripts in both lanes originate from *Eya2* morpholino-free cells that were isolated together with *Eya2* morpholino containing cells. This is unavoidable, since electroporation results in mosaic misexpression. Deletion of exons is not seen with control morpholinos (top row, lanes 3 and 7). Morpholinos do not affect the expression of the housekeeping gene cS17 (bottom row). +, RNA with reverse transcriptase; −, reverse transcriptase free control. (B) Both Eya2 morpholinos were electroporated into the future otic territory at stage HH5 and the expression of *Pax2* (blue) determined at stage HH11. Cells carrying the morpholinos were visualised by anti-Fluorescein antibodies (brown). No effect on otic placode formation was observed (arrowhead). (C, C′) Morpholinos targeting the ATG of Pax2 (fluorescent cells in C; see also D–D″) do not lead to up-regulation or loss of *Sox3* (C′); inset: overlay of fluorescent and bright field image. (D–D″, F–G″) Two different morpholinos were used to reduce Pax2 expression: the first morpholino disrupts translation initiation of Pax2 by targeting the translational start site of the transcript. The second morpholino was designed to interfere with splicing events at the exon 2–intron 3 boundary. Antibody staining against Pax2 reveals that both morpholinos effectively knock-down Pax2 (arrowheads in D–D″ and F–F″; Pax2ATG MO, ATG targeting morpholino; G–G″; Pax2Exon2 MO, exon 2–intron 3 targeting morpholino). (E–E″, H–H‴) Loss of Pax2 is not seen when sense control morpholinos are applied. op, otic placode; MO, morpholino. The bracket in B shows the area depicted in D–E″; Bars in D and E 100 μm.

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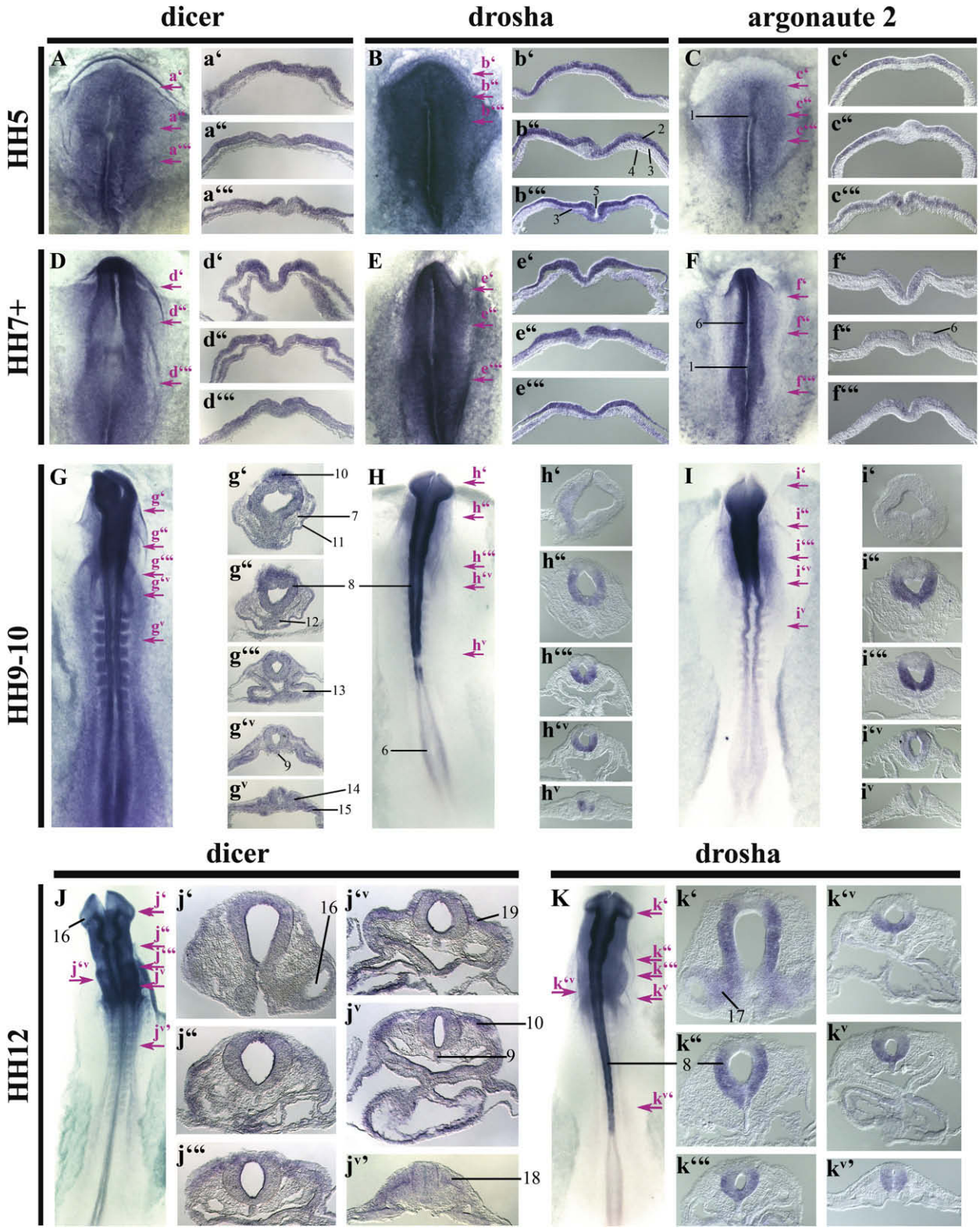

Fig. 4 – Expression patterns of *Dicer*, *Drosha* and *Argonaute2*. Expression of *Dicer* (A, D, G, J), *Drosha* (B, E, H, K) and *Argonaute2* (C, F, I) is shown for stages HH5 (A–C), HH7^{+} (D–F), HH9–10 (G–I). Expression at stage HH12 is shown for *Dicer* (J) and *Drosha* (K). Expression of *Argonaute2* is almost identical to that of *Drosha* at this stage (not shown). Section planes are indicated by small arrows. For details see text. 1, Hensen's node; 2, ectoderm; 3, mesoderm; 4, endoderm; 5, primitive grove; 6, neural plate; 7, head mesoderm; 8, neural tube; 9, notochord; 10, neural crest cells; 11, head ectoderm; 12, prechordal mesoderm; 13, heart; 14, somite; 15, lateral plate mesoderm; 16, optic vesicle; 17, optic stalk; 18, dermomyotome; 19, otic cup.

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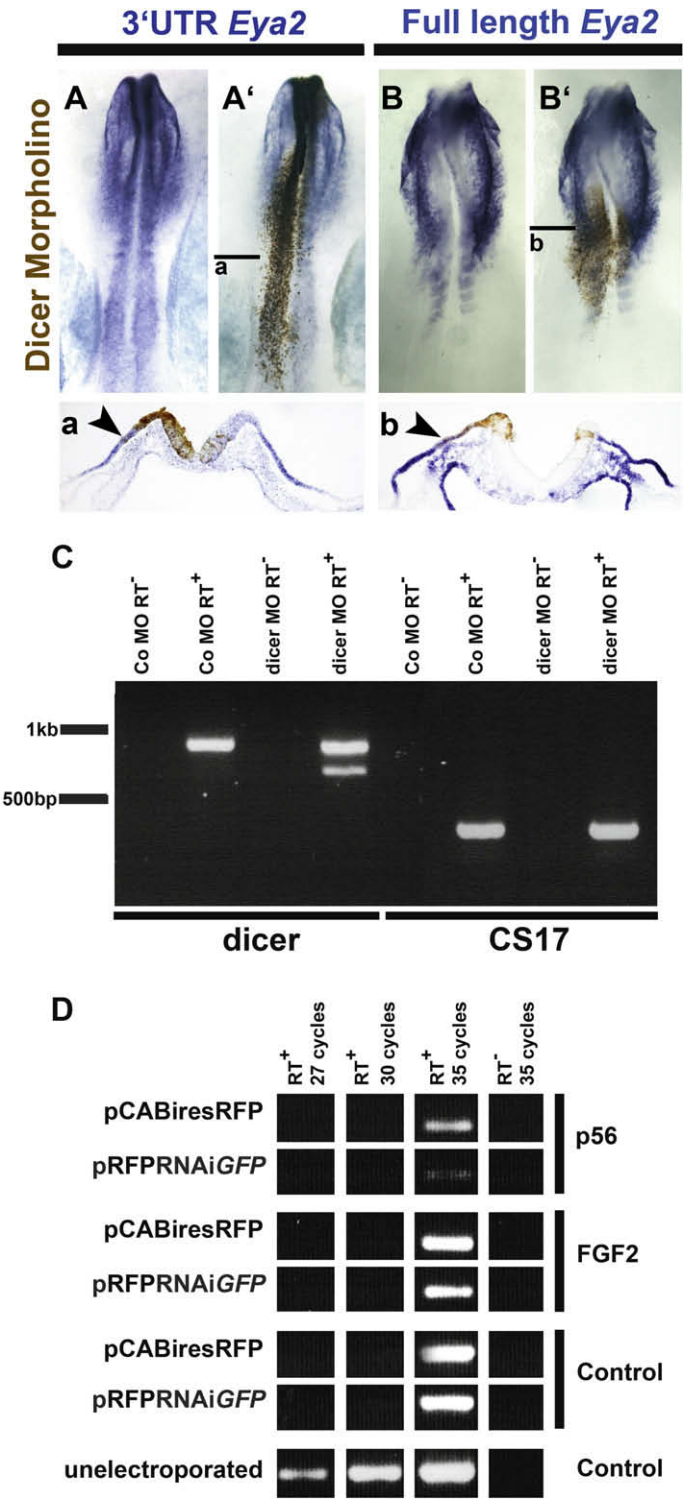
are up-regulated following ds/shRNA treatment (Sledz and Williams, 2005).

Using a RT-PCR approach we examined the expression of *p56* and *FGF2* in chick tissue electroporated with the gene silencing vector pRFPRNAi *GFP* or the control vector pCAβ-IRES-RFP. Amplicons for both transcripts are first detectable after the same number of PCR cycles irrespective of the vector. However, rather than up-regulation of *p56* or *FGF2* in the presence of the gene silencing vector we observed a slight reduction when compared with tissues expressing the control vector (Fig. 5D). These results suggest that electroporation of the gene silencing vectors is unlikely to activate the interferon-stimulated genes *p56* and *FGF2*.

2.6. *pRFPRNAi GFP expression activates the p53 pathway and causes increased apoptosis*

It has previously been reported that siRNA in mammalian cells and morpholinos in zebrafish (Robu et al., 2007; Scacheri et al., 2004) show off-target effects that lead to activation of



the p53 pathway and increased apoptosis. We therefore investigated whether expression of pRFPRNAi *GFP* induces apoptosis using the TUNEL method. About 50% of pRFPRNAi *GFP* electroporated embryos show an accumulation of cells undergoing apoptosis (12/26; Fig. 6B and B′). In contrast, when embryos expressing the control vectors pCAβ-IRES-RFP ($n = 11$, Fig. 6A and A′), pCAGGSeGFP ($n = 5$, not shown) or the pRFPRNAi backbone alone ($n = 7$, not shown) were analysed, no enhanced apoptosis was detected in electroporated tissues.

In the absence of cellular stress, p53 protein is expressed at low steady-state levels and exerts little, if any, effect on cell fate (Ashcroft et al., 2000; Wang et al., 2003). However, cellular stress can enhance its stability resulting in the accumulation of p53 protein (Bellamy et al., 1995; Hainaut, 1995; Yonish-Rouach, 1996). We therefore analysed p53 expression in pRFPRNAi *GFP* expressing tissue. We find that while low level of p53 protein is found in most cells, high expression levels are present in cells containing the GFP targeting pRFRNAi vector (Fig. 6C–C″). In contrast, expression of the pRFPRNAi vector backbone alone (0/7, Fig. 6E–E″), morpholinos (0/5, not shown) or pCAGGSeGFP (0/5, not shown) does not result in p53 accumulation. RT-PCR analysis of tissue electroporated with pRFPRNAi *GFP*, pCAβ-IRES-RFP, standard control or *Eya2* morpholinos shows that the p53 target genes *PIG3* (Flatt et al., 2000; Polyak et al., 1997), chick *MDM2* (*CDM2*; LaFleur et al., 2002) and *CIP1/p21* (Rahman-Roblick et al., 2007; Riley et al., 2008) are up-regulated in the presence of pRFPRNAi *GFP*, but not in any other tissue (Fig. 6D). These results suggest that the morphological defects and gene mis-regulation observed with pRFPRNAi vectors is due to activation of the p53 pathway, which leads to apoptosis via PIG3 and CDM2 (Bose and Ghosh, 2007; Contente et al., 2002; LaFleur et al., 2002; Polyak et al., 1997) and cell cycle arrest via CIP1/p21 (Sherr and Roberts, 1995; Waldman et al., 1995).

3. Discussion

Here we have evaluated different sequence-specific knock-down strategies in young chick embryos. We find that pRFPRNAi vectors, which use the cell intrinsic miRNA processing machinery to produce silencing RNAs cause non-specific, off-target effects. Their expression in chick ectoderm results in target independent mis-regulation of gene expression and in morphological defects. Our results propose that activation of the p53 pathway is at least partially responsible through induction of CIP1/p21 and the pro-apoptotic genes CDM2 and PIG3. In contrast, morpholinos specifically reduce gene expression, but do not cause non-specific effects observed with pRFPRNAi vectors. Our data suggest that morpholinos are preferable for knock-down experiments in early chick embryos.

3.1. *Involvement of the Dicer pathway?*

The gene silencing system introduced by Das and colleagues (2006) was designed to allow processing of vector-derived transcripts by the same cellular machinery that is required for the processing of endogenous miRNA transcripts. The major components of this pathway include the ribonuclease-III family enzymes Drosha and Dicer, the nuclear export proteins Exportin-5 and RanGTP, and the RISC component Argonaute2. In the multi-step process yielding the mature miRNA, primary miRNA precursors (pri-miRNA) are first converted to pre-miRNA through cleavage by Drosha within the nucleus. Exportin-5 and RanGTP export pre-miRNA into the cytoplasm, where Dicer cuts it into 18–22 nt duplexes. One strand of the mature miRNA now enters the protein effector RISC, which mediates the degradation or translation inhibition of mRNA targets (Carrington and Ambros, 2003; Murchison and Hannon, 2004; Pillai, 2005). The shRNA transcripts of the gene silencing vector are thought to undergo the same enzymatic cleavage described above to yield better silencing efficiency than expression of conventional double stranded RNA silencing molecules (Chang et al., 2006). However, if any of the major pathway members are not present, exogenous shRNA may be processed inappropriately and cause toxic effects. This possibility is unlikely, since we show that the three main effectors of the miRNA pathway, *Dicer*, *Drosha* and *Argonaute2*, are expressed in the ectoderm of stage HH5–HH8 embryos.

At later stages, only *Dicer* is expressed in all tissues studied (otic placode, otic cup and neural tube), while *Drosha* and *Argonaute2* are only detected in the neural tube. While it is possible that Drosha and Argonaute2 proteins remain stable once expressed in the early ectoderm (HH5–8), it is equally likely that at later stages Dicer associates with a different RISC to repress mRNA expression (Forstemann et al., 2007; Okamura et al., 2004; Tomari et al., 2007). Nonetheless, our results suggest that components of the miRNA pathway are differentially expressed, which should be taken into account when designing shRNA-mediated knock-down experiments.

We speculated that Dicer may be expressed at a rate-limiting level, and that introduction of the vectors may cause

Fig. 5 – Phenotypes caused by electroporation of pRFPRNAi vectors cannot be mimicked by altered *Dicer* expression and are not a consequence of IFN pathway activation. (A and B) Embryos electroporated with Dicer morpholino (brown) do not show any changes in *Eya2* expression (blue; A, A′, a: 3′UTR probe; B, B′, b, full length probe) unlike embryos expressing gene silencing vectors (compare Figs.1B and 2A, B). Small bars in A′ and B′ indicate the level of the sections shown in a and b, respectively. Arrowheads indicate cells that carry Dicer morpholino and express *Eya2* at levels comparable to the non-electroporated control side. (C) The standard control morpholino (CoMo) does not change the size of the *Dicer* amplicon (lane 2). In contrast, Dicer morpholinos lead to the excision of exon 8 as determined by the presence of a band 169 bp smaller than the wild type amplicon (lane 4). Neither morpholino affects the expression of the housekeeping gene *cS17* (lanes 5–8). Lanes 1, 3, 5 and 7 show control RNA without reverse transcriptase. (D) Expression of pRFPRNAi*GFP* in early chick embryos does not lead to an increase of the interferon responsive genes *p56* and *FGF2*, but may slightly decrease their expression compared to control tissue. Expression of the interferon pathway unrelated *Dicer* is unchanged under either experimental condition (Control). The bottom row shows the amplification of *Dicer* using cDNA from non-electroporated embryos as template.

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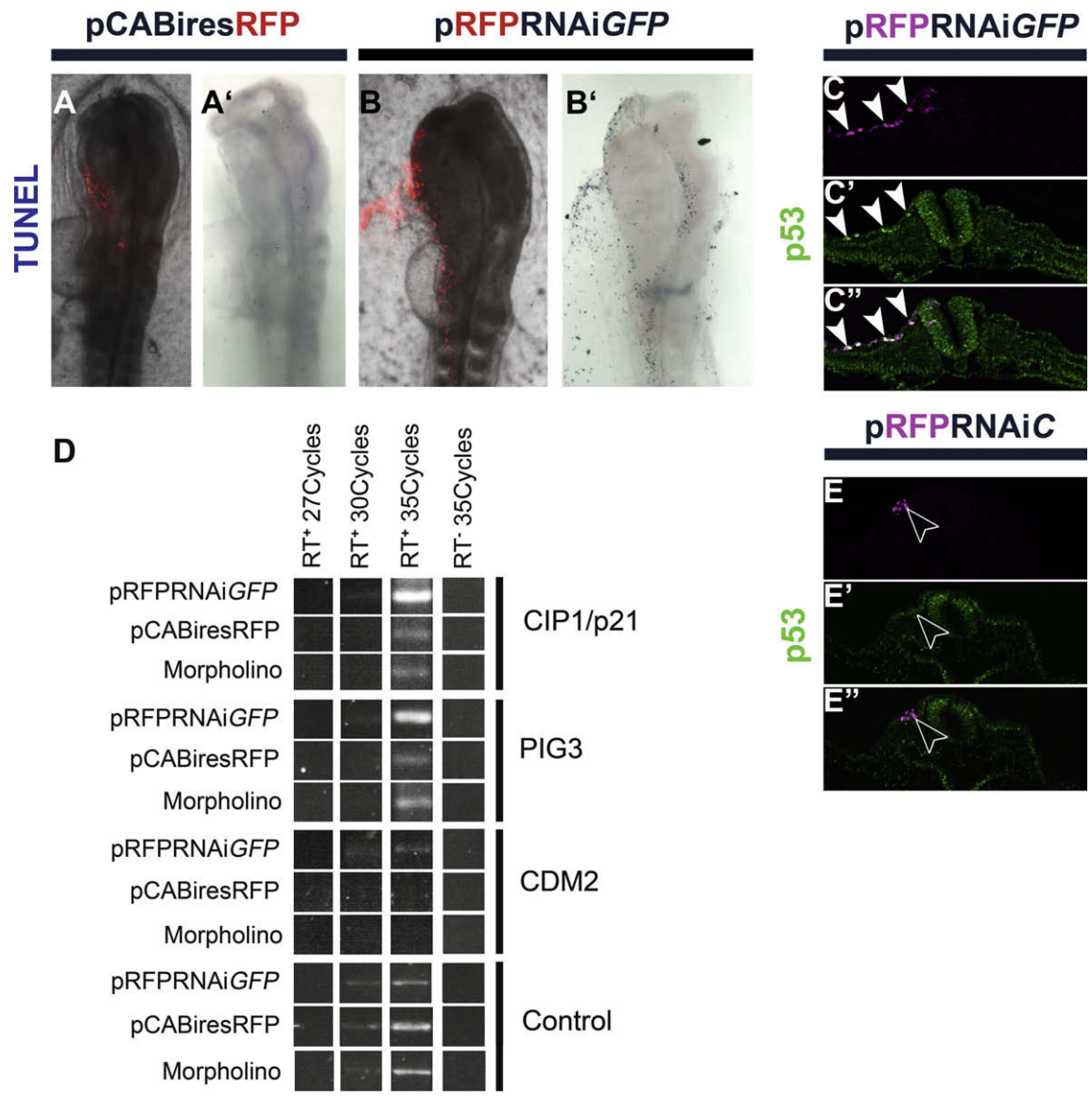

Fig. 6 – Electroporation of the gene silencing vector pRFPRNAi results in elevated levels of apoptosis, p53 stability and activation of p53 targets. (A and B) Cells expressing the gene silencing vector (B, red) undergo apoptosis (B′), while cells expressing RFP alone do not (A, red; A′). (C–C″). Electroporation of pRFPRNAi (visualised by expression of reporter RFP, C and C″, magenta) is accompanied by higher levels of p53 protein (arrowheads in C and C′, green; *n* = 4). An accumulation of p53 is not observed after electroporation of the pRFPRNAiC vector backbone (E–E″; *n* = 7). (D) Electroporation of gene silencing vectors into early chick embryos leads to increased expression of the p53 target genes *CIP1/p21, PIG2 and CDM2* when compared to tissue expressing RFP or morpholino (faint bands can already be discerned after 30 PCR cycles, stronger bands are clearly visible after 35 PCR cycles). The expression of p53 pathway unrelated *Dicer* under either experimental condition is shown in the bottom row (Control).

competition with endogenous miRNA processing events that require this enzyme. However, Dicer knock-down with a morpholino does not mimic the consequences of gene silencing vectors, and the vectors themselves do not affect *Dicer* expression. Taken together, these results suggest that the non-specific effects of pRFPRNAi vectors are independent of the Dicer pathway.

3.2. *Involvement of the interferon pathway?*

Although the interferon pathway has been implicated in mammalian innate immune response to small interfering RNA (Judge and Maclachlan, 2008; Sledz et al., 2003), similar effects have so far not been described in the chick (Chesnutt and Niswander, 2004). Our results do not provide support for the idea that ligand-independent activation of this pathway is a major cause of the non-specific pRFPRNAi effects. The expression of two major interferon responsive genes is not increased in tissues electroporated with the gene silencing vector. Recent studies reported that siRNA transfection results in the induction of only a subset of the 850 putative interferon-stimulated genes (Sledz et al., 2003). Since we do not know all of the responsive genes, the possibility of interferon mediated responses to pRFPRNAi expression cannot be excluded.

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Table 2 – List of oligoprimers used in RT-PCR experiments

Gene	Accession No.	Primer pair
Eya2 (E3–I4)	ENSGALG00000004508	F: TCACCCAGCCTGACTGTAAA R: GAAGGGCTGATGCTGTTTGT
Eya2 (E6–I7)	ENSGALG00000004508	F: TGCTGCATACACTGCCTACC R: ATCCGACCCTCTGTGATGTC
Dicer (E8–I9)	NP_001035555.1	F: GCAAGTGTCAGCTGTCAGGA R: ATTTGCGCAAGATTTCAAGC
FGF2	NM_205433	F: ATTGCTGGCACTGAAATGTG R: AGCTTTCGTTCCAGGTCCAG
P56	XM_426294	F: GGTGGTGGCTGTGAAAAAGT R: GGCTCTCCTGTGAGCATTTC
cS17	X07257	F: AGAAGGCGGCGCGGGTGATCATCG R: GTTTATTGTAAAAGCAACATAACG
PIG3	ENSGALT00000026619	F: TGTGCTGCCGTATTTCTCTG R: GGGTGTTTTCACTGGCATTT
CIP1/p21	NM_204396.1	F: GGAAGGGACTGAGGAGGACT R: GTGAGGCTCTGAGGGTTCTG
CDM2	AF005045	F: CAATCAACAAGACTCTACGCTGGCTG R: TCATCTTCATCTGTGAGCTCCTGTCC

F, forward primer; R, reverse primer. All primer sequences in 5′–3′ orientation.

3.3. *p53 activation and cell death as a consequence of pRFPRNAi vector expression*

Previous studies have demonstrated off-target effects caused by introduction of siRNA and morpholinos in different experimental systems. In zebrafish, about 15% of morpholinos have been reported to cause neural cell death, which in turn is attenuated by p53 knock-down (Ekker and Larson, 2001; Robu et al., 2007), suggesting that activation of the apoptotic pathway via p53 is involved in mediating such effects. Likewise, siRNA can cause changes in untargeted proteins like p53 and p21 (Scacheri et al., 2004). Our results show that in young chick embryos introduction of pRFPRNAi vectors targeting a variety of genes including GFP leads to morphological defects, increased apoptosis, the stabilisation of p53 protein and the activation of its targets *CDM2*, *PIG3* and *CIP1/p21*. CDM2 and PIG3 are known mediators of the apoptotic pathway (Bose and Ghosh, 2007; Contente et al., 2002; LaFleur et al., 2002; Polyak et al., 1997) and their activation is likely to account for the increased cell death observed. However, this increase does not appear to be sufficient to account for the complete loss of otic morphology and the dramatic changes in gene expression. We therefore suggest that in addition CIP1/p21-mediated cell cycle arrest is responsible for the non-specific effects observed.

4. Conclusion

Unlike pRFPRNAi, the morpholinos tested in this study do not show non-specific side-effects or activation of the p53 pathway. We have used five different morpholinos to alter the processing of newly synthesised transcripts (Schmajuk et al., 1999). Two of them were designed to interfere with splicing events at different exon–intron boundaries of the primary mRNA of the nuclear factor *Eya2*. Another morpholino was designed to target splicing events in *Dicer* transcripts causing a frame-shift and generating a premature stop codon. Finally, Pax2 morpholinos targeted both the transcription start site and intron–exon junction. The use of fluorescein-conjugated morpholinos allows their visualisation within cells immediately after electroporation (and also appears to be required for successful electroporation; Voiculescu et al., 2008). Although the morpholino is diluted due to cell division and disposal from cells (Heasman, 2002), we were able to detect strong fluorescence for at least 24 h after electroporation. The morpholinos used in this study showed effective excision of the targeted exons, did not affect other transcripts or cell survival, nor was exon excision observed using standard control or sense morpholinos. Although we have not systematically tested the difference between pRFPRNAi or morpholino based knock-down strategies at later stages, we find that introduction of pRFPRNAi silencing vectors in the neural tube of HH11–12 embryos causes transcript mis-regulation similar to the effects observed in young embryos (Fig. S1). This observation suggests that the vector-based silencing system needs to be accompanied by careful controls also at later stages. In summary, we conclude that morpholinos are effective and specific and represent the preferred approach for knock-down studies in young chick embryos.

5. Experimental procedures

5.1. *Embryo culture and electroporation*

Fertile hens' eggs (Winter Egg Farm, Hertfordshire, UK) were incubated in a humidified incubator at 38 °C and staged according to Hamburger and Hamilton (HH; Hamburger and Hamilton, 1951). Expression-vectors and fluorescein-coupled

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morpholinos were introduced into stage HH11 embryos using *in ovo* electroporation or into stage HH 4–6 embryos maintained in New culture (New, 1955; Stern and Ireland, 1981) using an electroporation chamber (McLarren et al., 2003).

For *in ovo* electroporation eggs were windowed and injected with Indian ink (1:20) to reveal the embryo. Approximately 0.5 μl of gene-specific or control RNAi vector (1 μg/μl in 6% sucrose containing 0.1% fast green) was injected into the lumen of the anterior neural tube. A current of 25 V (5 pulses, 25 ms duration, 1 s interval) was applied across electrodes placed 3–4 mm apart on either side of the embryo using an IntraCel ISS10 pulse generator. After electroporation windows were sealed with insulation tape and the eggs incubated for 20 h.

Stages 4–6 embryos attached to the vitelline membrane were placed into an electroporation chamber (McLarren et al., 2003) and approximately 0.5 μl vector DNA (1 μg/μl in 6% sucrose containing 0.1% fast green) or morpholino (0.7 μM in 6% sucrose, 0.1% fast green containing 50 ng/μl pCAβ-IRES-RFP DNA as 'carrier') was introduced between the vitelline membrane and the ectoderm. Using an IntraCel ISS10 pulse generator a current of 6 V (4 pulses, 50 ms duration, 1 s interval) was applied across the electrodes. After electroporation, embryos were maintained in New culture for 6–15 h at 38 °C in a humid atmosphere.

After incubation, embryos were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde and 2 mM EGTA for 4 h at room temperature before processing for *in situ* hybridisation.

5.2. *Whole mount in situ hybridisation, immunohistochemistry and TUNEL*

Embryos were processed for whole mount *in situ* hybridisation using digoxigenin (DIG)-labelled antisense RNA probes as previously described (Streit et al., 1998). The DIG-labelled antisense riboprobes were generated from linearised plasmids encoding chick Argonaute2 (ChEST 531J17, ARK-Genomics, UK; Boardman et al., 2002), BMP7 (Liem et al., 1995), Dicer (ChEST716m22, ARK-Genomics), Dlx5 (Ferrari et al., 1995), Drosha (ChEST1000b6, ARK-Genimics), Eya2 (Mishima and Tomarev, 1998), GATA3 (Sheng and Stern, 1999), GnRH (a gift from Dr. Ian Dunn), Notch1 (Myat et al., 1996), Pax2 (a gift from M. Goulding), RALDH3 (a gift from Malcolm Maden), Six1 (a gift from Gillermo Oliver), Six4 (Esteve and Bovolenta, 1999), Sox2 and -3 (a gift from Paul Scotting). NBT/BCIP (Roche, Switzerland) were used as substrates to detect transcripts. Stained embryos were photographed using an Olympus SZX12 stereomicroscope and an AxioCamHR digital camera (Zeiss, Germany).

Morpholinos were detected by fluorescence or by using horseradish peroxidase (POD) conjugated monoclonal antibodies against fluorescein (Roche), while GFP expressing cells were revealed using polyclonal anti-GFP antibodies (1:1000; Molecular Probes). Immunofluorescence on whole mounts and cryosections was performed as previously described (Bhattacharyya et al., 2004; McLarren et al., 2003), using polyclonal antibodies against mouse Pax2 (1:50; Zymed), monoclonal antibodies against Pax3, Pax6 (both Developmental Hybridoma Bank; 1:20) and p53 (Mab240 and Mab241, applied simultaneously, 1:100). Immunofluorescence staining with p53 was performed as described in Krinka et al., 2001. The appropriate Alexafluor 488 (Molecular Probes) and Cy5 (Invitrogen) secondary antibodies were used in a 1:1000 dilution; nuclei were stained by DAPI (Molecular Probes). Cryosections were examined and photographed using a Zeiss Axiovert 200M, ORCA digital camera (Hamamatsu) and SimplePCI software (Digital Pixel, UK) or a Leica TCS SP5 confocal microscope.

The terminal deoxynucleotidyl transferase-mediated dUTP-DIG nick end labelling (TUNEL) method to reveal cells undergoing apoptosis was performed on fixed whole mount embryos. Embryos were rinsed in PBS containing 0.1% Tween (PBT), washed in PBT for 1 h and treated with 10 μg/ml Proteinase K in PBT for 20 min. Embryos were then post-fixed for 30 min in 4% formaldehyde, 0.1% glutaraldehyde in PBT, washed for 1 h in PBT and incubated for 30 min in terminal deoxynucleotidyl transferase (TdT) buffer (Invitrogen). This was followed by an overnight incubation of the embryos in TdT buffer containing 20 mM DIG-dUTP (Boehringer–Mannheim) and 50 U of TdT enzyme (Invitrogen) at room temperature. Embryos were washed twice in PBT for 1 h at 65 °C and four times for 1 h at room temperature. Detection of the DIG-label was carried out following the *in situ* hybridisation protocol (Streit et al., 1998).

5.3. *Vectors, morpholinos and primers*

The RNAi vectors pRFPRNAi C, pRFPRNAi c*Notch1* and pRFPRNAi *GFP* (Das et al., 2006) were supplied by ARK-Genomics, UK. RNAi target finder at https://genscript.com/ssl-bin/app/rnai was used to select target sequences for Eya2 (G/ATACCTTCCTACAGCATCAAA), and Pax2 (G/TCCTCGGTCGAATG GCGAGAA). Target sequences were cloned into the first hairpin site of the pRFPRNAi C silencing vector according to the ARK-Genomics protocol. All oligoprimers used (for sequences see protocol supplied by ARK-Genomics, UK; Das et al., 2006) were synthesised and supplied by Invitrogen. The avian expression vectors pCAβ-IRES-mGFP/RFP (McLarren et al., 2003) and pCAGGSmCherry/eGFP (a kind gift from K. Langenfeld) were used as control vectors.

To down-regulate *cDicer1* (NP_001035555.1), fluorescein-coupled morpholino (ACAGCTTACAACCTTACCTGTTTAG; Gene Tools, USA) targeting the exon 8–intron 9 boundary of *cDicer1* were used. To reveal exon 8 excision (Fig. 5C), RT-PCR was performed using primers spanning exon 5–exon 9 (Table 2). The fluorescein-coupled standard control morpholino (Gene Tools, USA) was used for control experiments. Fluorescein-coupled morpholinos against *Eya2* (ENSGALG00000004508) were designed to interfere with splicing events at boundaries of exon 3–intron 4 (CTTGGCAGGAACACTCACTTTGTTG) and exon 6–intron 7 (ATAAATGCTGAGA TATACCTGATGC), resulting in deletion of exon 3 and exon 6, respectively (Fig. 1A). As control sense morpholinos and the standard control morpholino were used. RT-PCR was carried out to reveal exon excision (Table 2). Fluorescein-coupled Pax2 morpholinos were generated to target the exon 2–intron 3 boundary (GCGGACTCGCCCTTACCTGTTTATG, using ENSGALT00000009124 Pax2 sequence) and the transcription start site (GGTCTGCCTTGCAGTGCATATCCAT, using NM_204793

Pax2 sequence), sense morpholinos were used as controls. Efficient knock-down of Pax2 was assessed by Pax2 antibody staining (Fig. 3D–H). To study expression of *FGF2*, *p56*, *PIG3*, *CDM2*, *p21* and cS17 we used primers listed in Table 2.

5.4. RT-PCR

Tissues expressing pRFPRNAi *GFP*, pCAβ-IRES-RFP, morpholinos targeting *Dicer*, *Eya2* or standard control morpholinos were excised from electroporated embryos after 16 h in culture. Electroporated ectoderm was visualised using a fluorescence stereomicroscope, freed from the underlying mesoderm using 20 μg/ml dispase (Roche) in Tyrode's saline and collected in Tri-reagent. RNA was extracted using the TriPure Isolation Kit (Roche) and reversed transcribed using random primers (Promega). PCR was carried out using 2 μl template and 0.3 U TaqPol (Promega) in a PTC-200 Peltier Thermal Cycler for 2 min at 94 °C, 35 cycles of 94 °C for 1 min, 1 min primer annealing temperature, and 45 s at 72 °C. The final cycle step was followed by 12 min at 72 °C.

Acknowledgments

We thank Dr. Claudio Stern for helpful discussions and critical reading of the article, Dr. M. Tavassoli for helpful advice and Drs. Paul Scotting, Paola Bovolenta, Guillermo Oliver and Malcolm Maden for reagents. This work was supported by the BBSRC, a Marie Curie Early Stage Fellowship and Guy's and St Thomas' Charitable Foundation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mod.2008.08.005.

REFERENCES

Abello, G., Khatri, S., Giraldez, F., Alsina, B., 2007. Early regionalization of the otic placode and its regulation by the Notch signaling pathway. Mech. Dev. 124 (7–8), 631–645.

Adam, J., Myat, A., Le Roux, I., Eddison, M., Henrique, D., Ish-Horowicz, D., Lewis, J., 1998. Cell fate choices and the expression of Notch, Delta and Serrate homologues in the chick inner ear: parallels with *Drosophila* sense-organ development. Development 125, 4645–4654.

Ashcroft, M., Taya, Y., Vousden, K.H., 2000. Stress signals utilize multiple pathways to stabilize p53. Mol. Cell. Biol. 20, 3224–3233.

Becker, D., Ciantar, D., Catsicas, M., Pearson, R., Mobbs, P., 2001. Use of pIRES vectors to express EGFP and connexin constructs in studies of the role of gap junctional communication in the early development of the chick retina and brain. Cell Commun. Adhes. 8, 355–359.

Bel-Vialar, S., Itasaki, N., Krumlauf, R., 2002. Initiating Hox gene expression: in the early chick neural tube differential sensitivity to FGF and RA signaling subdivides the HoxB genes in two distinct groups. Development 129, 5103–5115.

Bellamy, C.O., Malcomson, R.D., Harrison, D.J., Wyllie, A.H., 1995. Cell death in health and disease: the biology and regulation of apoptosis. Semin. Cancer Biol. 6, 3–16.

Bhattacharyya, S., Bailey, A.P., Bronner-Fraser, M., Streit, A., 2004. Segregation of lens and olfactory precursors from a common territory: cell sorting and reciprocity of Dlx5 and Pax6 expression. Dev. Biol. 271, 403–414.

Boardman, P.E., Sanz-Ezquerro, J., Overton, I.M., Burt, D.W., Bosch, E., Fong, W.T., Tickle, C., Brown, W.R., Wilson, S.A., Hubbard, S.J., 2002. A comprehensive collection of chicken cDNAs. Curr. Biol. 12, 1965–1969.

Bose, I., Ghosh, B., 2007. The p53-MDM2 network: from oscillations to apoptosis. J. Biosci. 32, 991–997.

Bron, R., Eickholt, B.J., Vermeren, M., Fragale, N., Cohen, J., 2004. Functional knockdown of neuropilin-1 in the developing chick nervous system by siRNA hairpins phenocopies genetic ablation in the mouse. Dev. Dyn. 230, 299–308.

Carrington, J.C., Ambros, V., 2003. Role of microRNAs in plant and animal development. Science 301, 336–338.

Chang, K., Elledge, S.J., Hannon, G.J., 2006. Lessons from nature: microRNA-based shRNA libraries. Nat. Methods 3, 707–714.

Chen, C.M., Cepko, C.L., 2002. The chicken RaxL gene plays a role in the initiation of photoreceptor differentiation. Development 129, 5363–5375.

Chesnutt, C., Niswander, L., 2004. Plasmid-based short-hairpin RNA interference in the chicken embryo. Genesis 39, 73–78.

Contente, A., Dittmer, A., Koch, M.C., Roth, J., Dobbelstein, M., 2002. A polymorphic microsatellite that mediates induction of PIG3 by p53. Nat. Genet. 30, 315–320.

Das, R.M., Van Hateren, N.J., Howell, G.R., Farrell, E.R., Bangs, F.K., Porteous, V.C., Manning, E.M., McGrew, M.J., Ohyama, K., Sacco, M.A., Halley, P.A., Sang, H.M., Storey, K.G., Placzek, M., Tickle, C., Nair, V.K., Wilson, S.A., 2006. A robust system for RNA interference in the chicken using a modified microRNA operon. Dev. Biol. 294, 554–563.

Daudet, N., Lewis, J., 2005. Two contrasting roles for Notch activity in chick inner ear development: specification of prosensory patches and lateral inhibition of hair-cell differentiation. Development 132, 541–551.

Duchaine, T.F., Wohlschlegel, J.A., Kennedy, S., Bei, Y., Conte Jr., D., Pang, K., Brownell, D.R., Harding, S., Mitani, S., Ruvkun, G., Yates 3rd, J.R., Mello, C.C., 2006. Functional proteomics reveals the biochemical niche of *C. elegans* DCR-1 in multiple small-RNA-mediated pathways. Cell 124, 343–354.

Ekker, S.C., Larson, J.D., 2001. Morphant technology in model developmental systems. Genesis 30, 89–93.

Esteve, P., Bovolenta, P., 1999. CSix4, a member of the six gene family of transcription factors, is expressed during placode and somite development. Mech. Dev. 85, 161–165.

Ferrari, D., Sumoy, L., Gannon, J., Sun, H., Brown, A.M., Upholt, W.B., Kosher, R.A., 1995. The expression pattern of the distal-less homeobox-containing gene Dlx-5 in the developing chick limb bud suggests its involvement in apical ectodermal ridge activity, pattern formation, and cartilage differentiation. Mech. Dev. 52, 257–264.

Flatt, P.M., Polyak, K., Tang, L.J., Scatena, C.D., Westfall, M.D., Rubinstein, L.A., Yu, J., Kinzler, K.W., Vogelstein, B., Hill, D.E., Pietenpol, J.A., 2000. p53-dependent expression of PIG3 during proliferation, genotoxic stress, and reversible growth arrest. Cancer Lett. 156, 63–72.

Forstemann, K., Horwich, M.D., Wee, L., Tomari, Y., Zamore, P.D., 2007. *Drosophila* microRNAs are sorted into functionally distinct argonaute complexes after production by dicer-1. Cell 130, 287–297.

Funahashi, J., Okafuji, T., Ohuchi, H., Noji, S., Tanaka, H., Nakamura, H., 1999. Role of Pax-5 in the regulation of a mid-hindbrain organizer's activity. Dev. Growth Differ. 41, 59–72.

Garcia-Martinez, V., Alvarez, I.S., Schoenwolf, G.C., 1993. Locations of the ectodermal and nonectodermal subdivisions

of the epiblast at stages 3 and 4 of avian gastrulation and neurulation. J. Exp. Zool. 267, 431–446.

Groves, A.K., Bronner-Fraser, M., 2000. Competence, specification and commitment in otic placode induction. Development 127, 3489–3499.

Hainaut, P., 1995. The tumor suppressor protein p53: a receptor to genotoxic stress that controls cell growth and survival. Curr. Opin. Oncol. 7, 76–82.

Hamburger, V., Hamilton, H.L., 1951. A series of normal stages in the development of the chick embryo. J. Morphol. 88, 49–92.

Hammond, S.M., Boettcher, S., Caudy, A.A., Kobayashi, R., Hannon, G.J., 2001. Argonaute2, a link between genetic and biochemical analyses of RNAi. Science 293, 1146–1150.

Heasman, J., 2002. Morpholino oligos: making sense of antisense? Dev. Biol. 243, 209–214.

Judge, A., Maclachlan, I., 2008. Overcoming the innate immune response to small interfering RNA. Hum. Gene Ther. 19, 111–124.

Katahira, T., Nakamura, H., 2003. Gene silencing in chick embryos with a vector-based small interfering RNA system. Dev. Growth Differ. 45, 361–367.

Kos, R., Tucker, R.P., Hall, R., Duong, T.D., Erickson, C.A., 2003. Methods for introducing morpholinos into the chicken embryo. Dev. Dyn. 226, 470–477.

Krinka, D., Raid, R., Pata, I., Karner, J., Maimets, T., 2001. In situ hybridisation of chick embryos with p53-specific probe and their immunostaining with anti-p53 antibodies. Anat. Embryol. (Berl.) 204, 207–215.

LaFleur, D.A., Kim, H., Farris, J., Foster, D.N., 2002. Expression of the chicken homologue of the mouse double minute 2 gene. Biochim. Biophys. Acta 1574, 277–282.

Liem Jr., K.F., Tremml, G., Roelink, H., Jessell, T.M., 1995. Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. Cell 82, 969–979.

McLarren, K.W., Litsiou, A., Streit, A., 2003. DLX5 positions the neural crest and preplacode region at the border of the neural plate. Dev. Biol. 259, 34–47.

Mikuma, T., Kawasaki, H., Yamamoto, Y., Taira, K., 2004. Overexpression of Dicer enhances RNAi-mediated gene silencing by short-hairpin RNAs (shRNAs) in human cells. Nucleic Acids Symp. Ser. (Oxf.), 191–192.

Mishima, N., Tomarev, S., 1998. Chicken eyes absent 2 gene: isolation and expression pattern during development. Int. J. Dev. Biol. 42, 1109–1115.

Muramatsu, T., Mizutani, Y., Ohmori, Y., Okumura, J., 1997. Comparison of three nonviral transfection methods for foreign gene expression in early chicken embryos in ovo. Biochem. Biophys. Res. Commun. 230, 376–380.

Murchison, E.P., Hannon, G.J., 2004. MiRNAs on the move: miRNA biogenesis and the RNAi machinery. Curr. Opin. Cell Biol. 16, 223–229.

Myat, A., Henrique, D., Ish-Horowicz, D., Lewis, J., 1996. A chick homologue of Serrate and its relationship with Notch and Delta homologues during central neurogenesis. Dev. Biol. 174, 233–247.

Nakamura, H., Katahira, T., Sato, T., Watanabe, Y., Funahashi, J., 2004. Gain- and loss-of-function in chick embryos by electroporation. Mech. Dev. 121, 1137–1143.

Nakamura, H., Watanabe, Y., Funahashi, J., 2000. Misexpression of genes in brain vesicles by in ovo electroporation. Dev. Growth Differ. 42, 199–201.

New, D.A.T., 1955. A new technique for the cultivation of the chick embryo in vitro. J. Embryol. Exp. Morphol. 3, 326–331.

Okamura, K., Ishizuka, A., Siomi, H., Siomi, M.C., 2004. Distinct roles for argonaute proteins in small RNA-directed RNA cleavage pathways. Genes Dev. 18, 1655–1666.

Papanayotou, C., Mey, A., Birot, A.M., Saka, Y., Boast, S., Smith, J.C., Samarut, J., Stern, C.D., 2008. A mechanism regulating the onset of Sox2 expression in the embryonic neural plate. PLoS Biol. 6, e2.

Pebernard, S., Iggo, R.D., 2004. Determinants of interferon-stimulated gene induction by RNAi vectors. Differentiation 72, 103–111.

Pillai, R.S., 2005. MicroRNA function: multiple mechanisms for a tiny RNA? RNA 11, 1753–1761.

Polyak, K., Xia, Y., Zweier, J.L., Kinzler, K.W., Vogelstein, B., 1997. A model for p53-induced apoptosis. Nature 389, 300–305.

Rahman-Roblick, R., Roblick, U.J., Hellman, U., Conrotto, P., Liu, T., Becker, S., Hirschberg, D., Jornvall, H., Auer, G., Wiman, K.G., 2007. p53 targets identified by protein expression profiling. Proc. Natl. Acad. Sci. USA 104, 5401–5406.

Riley, T., Sontag, E., Chen, P., Levine, A., 2008. Transcriptional control of human p53-regulated genes. Nat. Rev. Mol. Cell Biol. 9, 402–412.

Robu, M.E., Larson, J.D., Nasevicius, A., Beiraghi, S., Brenner, C., Farber, S.A., Ekker, S.C., 2007. p53 activation by knockdown technologies. PLoS Genet. 3, e78.

Scacheri, P.C., Rozenblatt-Rosen, O., Caplen, N.J., Wolfsberg, T.G., Umayam, L., Lee, J.C., Hughes, C.M., Shanmugam, K.S., Bhattacharjee, A., Meyerson, M., Collins, F.S., 2004. Short interfering RNAs can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells. Proc. Natl. Acad. Sci. USA 101, 1892–1897.

Schmajuk, G., Sierakowska, H., Kole, R., 1999. Antisense oligonucleotides with different backbones. Modification of splicing pathways and efficacy of uptake. J. Biol. Chem. 274, 21783–21789.

Sheng, G., dos Reis, M., Stern, C.D., 2003. Churchill, a zinc finger transcriptional activator, regulates the transition between gastrulation and neurulation. Cell 115, 603–613.

Sheng, G., Stern, C.D., 1999. Gata2 and Gata3: novel markers for early embryonic polarity and for non-neural ectoderm in the chick embryo. Mech. Dev. 87, 213–216.

Sherr, C.J., Roberts, J.M., 1995. Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev. 9, 1149–1163.

Sledz, C.A., Holko, M., de Veer, M.J., Silverman, R.H., Williams, B.R., 2003. Activation of the interferon system by short-interfering RNAs. Nat. Cell Biol. 5, 834–839.

Sledz, C.A., Williams, B.R., 2004. RNA interference and double-stranded-RNA-activated pathways. Biochem. Soc. Trans. 32, 952–956.

Sledz, C.A., Williams, B.R., 2005. RNA interference in biology and disease. Blood 106, 787–794.

Stern, C.D., Ireland, G.W., 1981. An integrated experimental study of endoderm formation in avian embryos. Anat. Embryol. (Berl.) 163, 245–263.

Streit, A., 2002. Extensive cell movements accompany formation of the otic placode. Dev. Biol. 249, 237–254.

Streit, A., Lee, K.J., Woo, I., Roberts, C., Jessell, T.M., Stern, C.D., 1998. Chordin regulates primitive streak development and the stability of induced neural cells, but is not sufficient for neural induction in the chick embryo. Development 125, 507–519.

Tomari, Y., Du, T., Zamore, P.D., 2007. Sorting of *Drosophila* small silencing RNAs. Cell 130, 299–308.

Tucker, R.P., 2001. Abnormal neural crest cell migration after the in vivo knockdown of tenascin-C expression with morpholino antisense oligonucleotides. Dev. Dyn. 222, 115–119.

Voiculescu, O., Bertocchini, F., Wolpert, L., Keller, R.E., Stern, C.D., 2007. The amniote primitive streak is defined by epithelial cell intercalation before gastrulation. Nature 449, 1049–1052.

Voiculescu, O., Papanayotou, C., Stern, C., 2008. Spatially and temporally controlled electroporation of early chick embryos. Nat. Protoc. 3, 419–421.

Please cite this article in press as: Mende, M. et al., Specific and effective gene knock-down in early chick embryos using morpholinos but not pRFPRNAi vectors ..., Mech. Dev. (2008), doi:10.1016/j.mod.2008.08.005

Waldman, T., Kinzler, K.W., Vogelstein, B., 1995. p21 is necessary for the p53-mediated G1 arrest in human cancer cells. Cancer Res. 55, 5187–5190.

Wang, W., Takimoto, R., Rastinejad, F., El-Deiry, W.S., 2003. Stabilization of p53 by CP-31398 inhibits ubiquitination without altering phosphorylation at serine 15 or 20 or MDM2 binding. Mol. Cell. Biol. 23, 2171–2181.

Witting, S.R., Brown, M., Saxena, R., Nabinger, S., Morral, N., 2008. Helper-dependent adenovirus-mediated short hairpin RNA expression in the liver activates the interferon response. J. Biol. Chem. 283, 2120–2128.

Yonish-Rouach, E., 1996. The p53 tumour suppressor gene: a mediator of a G1 growth arrest and of apoptosis. Experientia 52, 1001–1007.

Please cite this article in press as: Mende, M. et al., Specific and effective gene knock-down in early chick embryos using morpholinos but not pRFPRNAi vectors ..., Mech. Dev. (2008), doi:10.1016/j.mod.2008.08.005